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ORIGINAL ARTICLE

C_{50} carotenoids extracted from *Haloterrigena thermotolerans* strain K15: antioxidant potential and identification

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Abstract

Halophilic archaea are one of the microorganism groups that have adapted to living in high saline environments and are important in terms of their potential use in biotechnology industry. One of the most important compounds they have, carotenoid, is used in food, cosmetics, and medical industries. The selected strain was identified as an extremely halophilic and thermophilic archaeon, *Haloterrigena thermotolerans* K15, based on morphological, biochemical, and physiological evidence as well as 16S rRNA analysis and screened by a scanning electron microscope and an atomic force microscope for the first time. The carotenoid contents of *H. thermotolerans* K15 isolated from Salt Lake (Tuz Gölü, Turkey) were determined by RP-HPLC–DAD and their isomers were characterized according to UV–Vis spectra by *cis* peak intensity and spectral fine structure. In addition to all-trans bacterioruberin as a major carotenoid, many isomers of the bacterioruberin such as monoanhydrobacterioruberin and bisanhydrobacterioruberin were also found. The antioxidant activity of carotenoid extract from *H. thermotolerans* was analyzed by the 2,2-diphenyl-1-picrylhydrazyl radical scavenging method. The carotenoid extract showed antioxidant activity statistically significantly higher than ascorbic acid and butylated hydroxytoluene as reference compounds ($p < 0.05$). This is the first study about carotenoid characterization and antioxidant activity of *H. thermotolerans* K15. The obtained results suggest the potential use of *H. thermotolerans* K15 products as a substitute for synthesized chemical carotenoids and antioxidants.

Keywords Bacterioruberin · Antioxidant · *Haloterrigena thermotolerans* · SEM · AFM

Introduction

Carotenoids are the most important natural pigments that can be obtained from a wide variety of organisms such as bacteria, archaea, fungi, plants, and algae. They have structural differences and functions and can have different colors, from yellow to red (Oliver and Palou 2000). Most of the members of the Halobacteriaceae family also have pink, orange, or red pigments due to their carotenoid content, and they may cause color changes in environments where they spread and breed widely. These pigments protect the cells against sun rays, radiation, heat, and evaporation in salts (Castillo et al. 2006). C_{50} carotenoids play critical biological roles in halophilic archaea, including acting as an antioxidant that protects cells from oxidative damage. Owing to the high concentration of conjugated double bonds in C_{50}, it has a far greater antioxidant activity than carotenoids such as beta-carotene (Giani et al. 2019; Flores et al. 2020). That is why halophilic archaea are immune to the deleterious effects of ultraviolet light and oxidative stress metabolites (Giani and Martínez-Espinosa 2020). The most considerable biological activities of carotenoids are as follows: provitamin A activity, antioxidant effect, cellular communication, immune-boosting effect, skin protection against UV, eye health-protecting effects, and wide usage in different industries as coloring and antioxidants, especially in cosmetics, feed, pharmaceutical, and food industries (Calegari-Santos et al. 2016). The carotenoids contain a large number

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of conjugated double bonds, allowing them to function as radical scavengers, thereby protecting against oxidative damage caused by harmful molecules such as reactive oxygen and nitrogen (Miller et al. 1996; Squillaci et al. 2017).

DPPH radical scavenging activity is a prevalent method in determining the antioxidant activity of carotenoids (Yachai 2009). In recent years, the search for suitable strategies to overcome oxidative stress mechanisms has become one of the important goals of biological research (Giani and Martínez-Espinosa 2020). Besides, carotenoids functioning as antioxidant agents play important roles as drug and food additives (Squillaci et al. 2017).

Increasing concerns about synthetic carotenoids produced by chemosynthesis that cause allergy, hypersensitivity, intolerance, and childhood hyperactivity have led to the rise of interest in the production of biological carotenoids (Yachai 2009). It is thought that the determination of antioxidant activities of carotenoids extracted from halophilic organisms will increase their usage areas in the food, cosmetics, and pharmaceutical industries as coloring.

Membrane stabilization in halophilic archaea is provided by C_{50} carotenoids, which have a polar end in their molecules and a suitable length for membrane stabilization. It has been determined that C_{50} carotenoids concerning the molecular length show a better binding rate with the archeal phytanyl lipids than C_{40} carotenoids (Kirti et al. 2014). In addition to bacterioruberin and its derivatives, which are major carotenoids in halophilic archaea and have 50 carbon atoms in their structure, some C_{40} carotenoids and C_{45} carotenoids such as 2-isopentenyl-3,4-dehydrorodopin are also synthesized in halophilic archaea (Oren et al. 2018; Giani et al. 2019). The propriety of extremely halophilic microorganisms to be able to grow in non-aseptic conditions and that carotenoids can be extracted from the cell without the need for mechanical disintegration represent an alternative in the industrial production of carotenoids.

The aim of the study was the biochemical and phylogenetic analysis of *Haloterrigena thermotolerans* K15 isolated from Salt Lake (Turkey), characterization of its carotenoids by chromatographic devices, and antioxidant property by DPPH radical scavenging method.

Materials and methods

Sample collection, isolation, and characterization of the strain

The salt samples were collected from Salt Lake, located in Tuzyaka, Konya, Turkey (38° 46′ 09.1″ N 33° 33′ 16.7″ E). The samples taken under aseptic conditions were dissolved in sterile 15% NaCl solution and 1 mL of resulted brine was mixed with 30 mL of molten agar medium JCM 168 which contained casamino acids (5 g/L), L-glutamic acid (1 g/L), yeast extract (5 g/L), trisodium citrate (3 g/L), $MgSO_4{\cdot}7H_2O$ (29.5 g/L), KCl (2 g/L), NaCl (175.5 g/L), $FeCl_2{\cdot}4H_2O$ (0.036 g/L), $MnCl_2{\cdot}4H_2O$ (0.36 mg/L). The medium pH was 7.0–7.2 before autoclaving. After the enrichment at 40 °C, for 2 weeks, samples were inoculated to MAM JCM 168 plates including 20 g/L agar (Enache et al. 2008). The pink-red pigmented colonies were selected for the study. Antibiotic susceptibility was tested by the disc diffusion method using bacitracin, novobiocin, erythromycin, ampicillin, vancomycin, penicillin, streptomycin, cefotaxime, tetracycline, and chloramphenicol discs. In addition to Gram staining, optimum NaCl concentrations, pH, and temperature range, minimum yeast, and Mg concentrations for growth, ability to grow on single carbon sources, nitrate reduction to nitrite, catalase, and oxidase tests, hydrolysis of starch, casein, gelatin, and Tween 80 tests were performed for strain K15 (Montalvo-Rodríguez et al. 2000).

DNA isolation from selected strain was carried out using the method reported by Neumann et al. (1992). The 16S rRNA genes of strain were amplified by PCR using the primers Arc7f′ 5′-TTCYGGTTGATCCYGCC-3′ and Arc1384r′ 5′-CGGTGTGTGCAAGGAGCA-3′. The sequences obtained were analyzed using NCBI Blast and aligned with other reported strains gene sequences. A phylogenetic tree was reconstructed by the neighbor-joining method. Sequence data of strain K15 was submitted to the NCBI database (GenBank) under accession number MW227318.

Screening of Haloterrigena thermotolerans by SEM and AFM

Fresh cultures of *H. thermotolerans* K15 incubated at 37 °C for 7 days were taken from the agar surface and applied to the coverslip surface slightly and treated overnight in a 2% glutardialdehyde solution after waiting to air dry. The preparations were removed from the solution and left to dry in the air before being dried in the acetone series of 30, 50, 70, and 90% for 10 min, and 30 min in 100% respectively in order to completely remove the water (Li et al. 2020). The preparations were placed on the Cressington Sputter Coater Au–Pd and coated with 40 mA for 60 s and then placed on the FEI Quanta FEG 250 SEM (operating at 10 kV) immediately. The same preparation technique was used in Bruker Edge3 AFM for three-dimensional imaging.

Carotenoid extraction and analysis

Carotenoid extraction and analysis were performed according to Squillaci et al. (2017) with some modifications. Cells were harvested on the seventh day of growth which is the beginning of the stationary phase to obtain the highest amount of biomass and prevent pigment loss due to potential cell lysis. The culture was centrifuged at 9000×*g* for 60 min at 4 °C and the supernatant was removed. The cell pellet was washed with phosphate-buffered saline (PBS) followed by centrifugation and was dried to constant weight under nitrogen gas. Two milliliters of 0.5% butylated hydroxytoluene (BHT) containing methanol was added to the dry cell pellet for the extraction of carotenoids and centrifuged at 9000×*g* for 60 min at 4 °C. All steps were carried out in the dim light. The total carotenoid amount of the strain was calculated by the extinction coefficient method and 2660 was used as $A_{1\mathrm{cm}}^{\%1}$ value (Britton 1985).

The carotenoid extract was primarily analyzed by thin-layer chromatography (TLC) with a silica gel plate (20 × 20 cm, Merck). In TLC, chloroform–methanol (93:7 v/v) was used as eluent, and spots were examined under visible light (Asker et al. 2002). The spots observed in TLC were recovered with methanol containing 0.2% BHT (Squillaci et al. 2017). Both spots and extract were analyzed in RP-HPLC (LC-20A Prominence, Shimadzu, Japan) with an LC-20AT quaternary pumps and SPD-M20A photodiode array detector at 490 nm. Characterization of the peaks was obtained according to the HPLC chromatogram of the total extract, UV–Vis spectrum, spectral fine structure, and intensity of the *cis* peaks and compared with published papers (Ronnekleiv 1995; Asker et al. 2002; Mandelli et al. 2012; Squillaci et al. 2017). Spectral fine structure (%III/II) and *cis* peak intensity ($\%A_B/A_{II}$) calculations are made based on maximum wavelength values. The %III/II value is obtained by dividing the highest wavelength absorption peak (III) and the middle absorption peak (II) by taking the minimum point between these two peaks as the starting point and multiplying by 100. The $\%A_B/A_{II}$ value is obtained by dividing the absorption value (A_B) of the highest *cis* peak by the middle absorption peak (A_{II}) and multiplying the result by 100 (Britton et al. 1995) (Fig. 1a, b).

Antioxidant activity measurements of carotenoids

DPPH radical scavenging activity method was used to determine the antioxidant activity of carotenoids. The culture incubated at 37 °C for 7 days in a 50-mL liquid medium was centrifuged at 9000×*g* for 60 min at 4 °C, and the supernatant was removed. The pellet was washed with PBS and after centrifugation, they were dried under nitrogen gas. The obtained carotenoid extract was diluted with methanol and 150 µL of the sample was mixed with 1.350 µL of 60-µmol/L DPPH solution prepared daily. Samples were loaded into microplate wells (Epoch 2, BioTek Instruments, Inc., USA) and after 30 min, an absorbance reading at 580 nm was taken against a blank containing absolute methanol. The antioxidant activities of ascorbic acid and butylated hydroxytoluene were measured in the same way. Besides, 150 µL of methanol and 1350 µL of DPPH solution were loaded into wells by mixing, and the DPPH radical scavenging activity of the samples was calculated as a percentage using the formula below. A_0 is the absorbance of the control and A_1 is the absorbance of the extract (Jiménez-Escrig et al. 2000).

$$\text{DPPH Radical Scavenging Activity }(\%) = [(A_0 - A_1)] \times 100$$

Statistical analysis

Data obtained from RSA analysis were tested with a two-way ANOVA with antioxidants and concentrations as the main effect, following one-way ANOVA and a post hoc Tukey test.

Results

Isolation and characterization of Haloterrigena thermotolerans strain K15

A halophilic archaeon, isolated from Salt Lake located in Tuzyaka, Konya, Turkey, is Gram-negative coccobacilli, with approximately 800 × 600-nm size measured by SEM and AFM (Figs. 2 and 3). The pigmentation of colonies is red. According to antibiotic susceptibility tests, the strain was susceptible to novobiocin and bacitracin antibiotics with 55- and 50-mm zones on the agar plate, respectively. Strain K15 was found extremely thermotolerant due to its

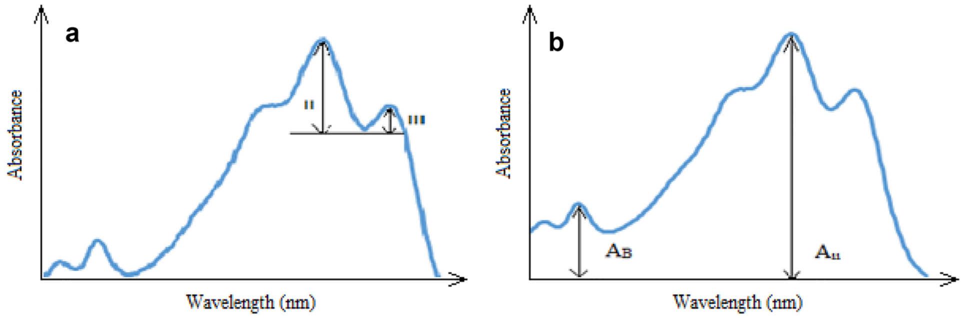


Fig. 1 Peak points of the spectrums using spectral fine structure (**a**) and *cis* peak intensity (**b**) calculations

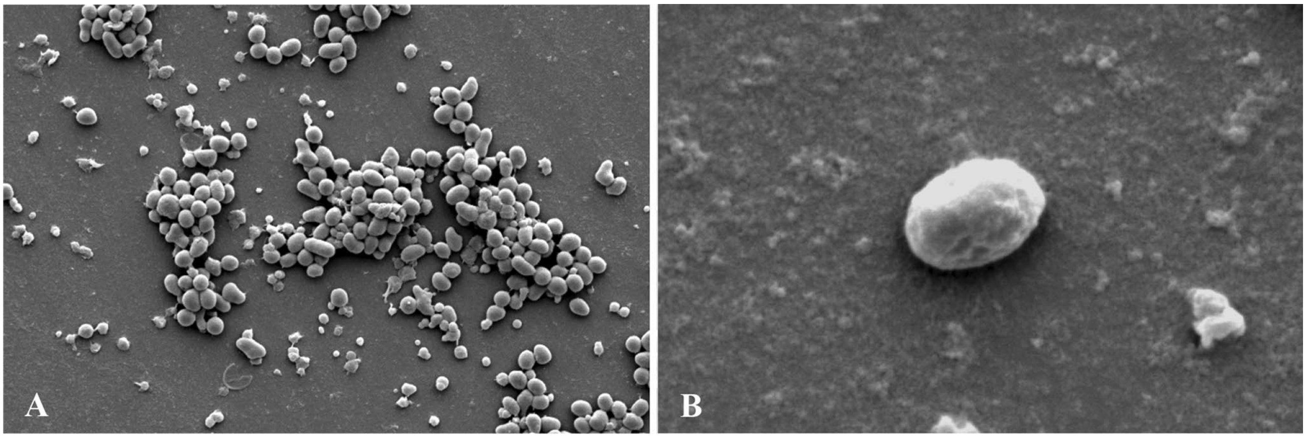
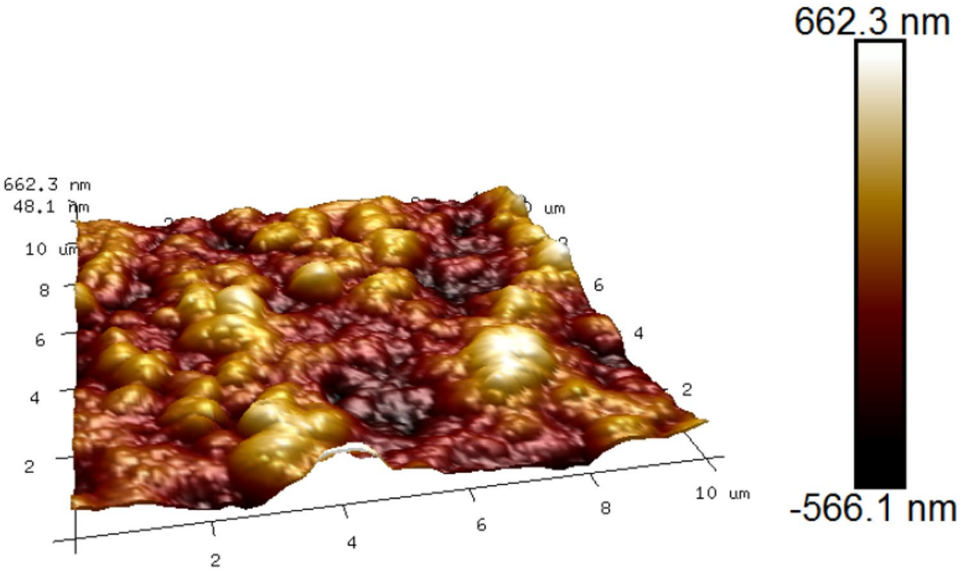


Fig. 2 Scanning electron microscope (FEI Quanta FEG 250 SEM) images of *Haloterrigena thermotolerans* K15 (**A** operating at 5 kV, 10.000×; **B** operating at 5 kV, 60.000×)

Fig. 3 Atomic force microscope (Bruker Edge3 AFM) image of *Haloterrigena thermotolerans* K15



ability to grow at 30–58 °C. It was able to grow at pH 6–7.5 and salt range 1.7–4.2 mol/L with optimum 3–3.5 mol/L. The minimum Mg requirement for growth was 4.8 mmol/L. While its oxidase reaction was negative, catalase reaction was found positive. While gelatinase and caseinase activities were detected, starch and Tween 80 were not hydrolyzed. Biochemical characterization revealed that the strain K15 utilize glucose and sucrose, but not lactose, maltose, fructose, mannitol, trehalose, and galactose. Nitrate reduction to nitrite was not observed. The strain exhibited levels of 16S rRNA similarity of 99.5% to the type strain of *Haloterrigena thermotolerans* PR5. Sequences of strain K15 were submitted to the NCBI database under the accession number MW227318. The phylogenetic tree was reconstructed based on the sequence obtained and its close relatives as well as *Methanospirillum hungatei* as an outgroup (Fig. 4).

Carotenoid extraction and characterization

The total amount of carotenoids produced by *H. thermotolerans* K15 was 21.43 μg/g dry cell. Carotenoid extract of *H. thermotolerans* was analyzed by a microplate reader (Epoch 2, BioTek Instruments, Inc., USA) with spectral scanning and a characteristic spectrum of bacterioruberin was obtained with the three peaks at 494, 527, and 467 nm with a broad shoulder and two *cis* absorption maxima at around 369 and 387 nm. Three pink spots were detected on the TLC silica gel plates. Each spot and the total extract were analyzed by HPLC and detected that each spot on TLC plates has multiple compounds. Fourteen peaks were detected on the chromatogram of total carotenoid extract. Each peak was examined in terms of maximum wavelengths and calculated the spectral fine structure and *cis* peak intensity. According

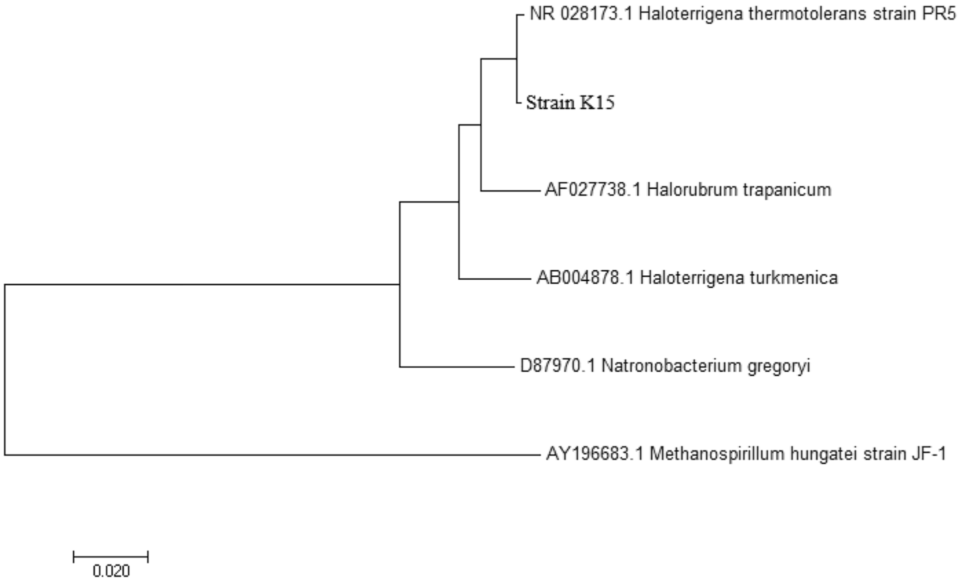


Fig. 4 Phylogenetic tree of *Haloterrigena thermotolerans* K15 and its close relatives. *Methanospirillum hungatei* was used as an outgroup

to all of these data, all-trans bacterioruberin was found as a major carotenoid of *Haloterrigena thermotolerans* K15 with maximum wavelengths at 369, 387, 467, 495,527, %III/II = 56.55, and %A_B/A_{II} = 12.56. Besides, five isomers of bacterioruberin were also detected: 9Z, 9′Z bacterioruberin, 15Z bacterioruberin, 5Z bacterioruberin, 9Z bacterioruberin, and 13Z bacterioruberin. Furthermore, four isomers of monoanhydrobacterioruberin (All E MABR, 5Z MABR, 9Z MABR, 13Z MABR) and four isomers of bisanhydrobacterioruberin (All E BABR, 9Z, 9′Z BABR, 9Z BABR, 13Z BABR) were detected as carotenoids of *H. thermotolerans* (Fig. 5, Table 1).

Antioxidant activity of the carotenoid extract

DPPH radical scavenging activity (RSA) methods were used to determine the antioxidant activity of carotenoids extracted from *H. thermotolerans* K15. Ascorbic acid and BHT were used as positive controls. Three different concentrations at 0.09, 0.45, and 0.90 µg/mL of the carotenoid extract, and

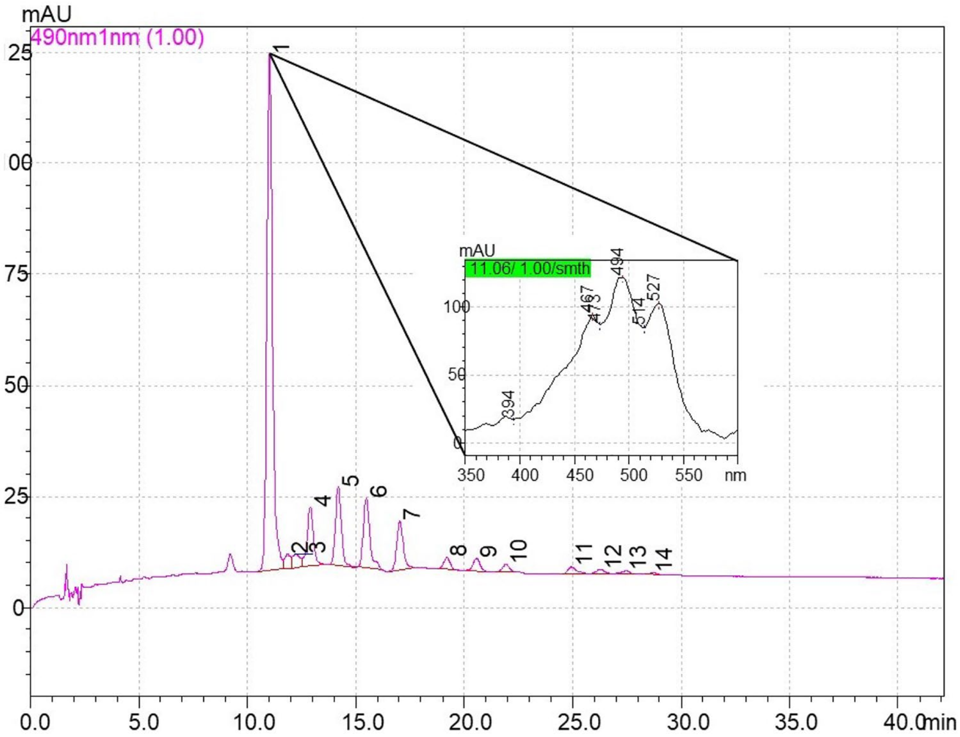


Fig. 5 HPLC (Shimadzu LC-20A Prominence) chromatogram of carotenoid extract from *Haloterrigena thermotolerans* K15 and spectrum scanning of the all-trans bacterioruberin

Table 1 HPLC–DAD peak's maximum absorbance, spectral fine structures, *cis* peak intensities, and peak retention times of whole carotenoids by *Haloterrigena thermotolerans* K15

Peak	Abs Max.[a]	III/II[b]	A_B/A_{II}[c]	R.T.[d]	C.T.[e]	References
1	369,387,467,495,527	56.55	12.56	11.018	All E BR	Mandelli et al. (2012)
2	368,387,460,487,516	42.87	35.30	11.864	9Z, 9′Z BR	Ronnekleiv (1995)
3	366,383,461,487,516	22.21	76.26	12.21	15 Z BR	Squillaci et al. (2017)
4	368,386,465,490,523	55.76	14.53	12.895	5 Z BR	Ronnekleiv (1995)
5	368,386,461,487,519	47.56	34.12	14.188	9 Z BR	Squillaci et al. (2017)
6	368,384,460,487,519	36.54	80.25	15.483	13 Z BR	Squillaci et al. (2017)
7	371,388,468,496,526	47.36	18.75	15.895	All E MABR	Ronnekleiv (1995)
8	365,387,490,523	39.85	45.23	19.195	5 Z MABR	Ronnekleiv (1995)
9	368,387,460,521	45.26	40.73	20.56	9 Z MABR	Squillaci et al. (2017)
10	368,386,459,488,519	35.84	87.90	21.926	13 Z MABR	Squillaci et al. (2017)
11	373,387,468.96,495,527	50.20	25.68	24.942	All E BABR	Ronnekleiv (1995)
12	366,387,464,488,519	25.63	35.96	26.285	9Z, 9Z′BABR	Ronnekleiv (1995)
13	368,387,467,488.8,518.56	42.60	40.96	27.462	9Z BABR	Squillaci et al. (2017)
14	368,387,487,518	27.56	82.63	28.655	13 Z BABR	Squillaci et al. (2017)

[a] *Abs Max.*, HPLC–DAD peak's maximum absorbance (nm)

[b] *%III/II*, spectral fine structures (%)

[c] *%AB/AII*, *cis* peak intensities (%)

[d] *R.T.*, retention time (min.)

[e] *C.T.* carotenoid type: *BR* bacterioruberin, *MABR* monoanhydrobacterioruberin; *BABR* bisanhydrobacterioruberin

positive controls were measured in four replicates to calculate the IC_{50} value of the carotenoid. The percentage rates of RSA belonging to *Haloterrigena thermotolerans* K15 extract were found as 8.75 ± 0.70, 24.27 ± 0.83, and 36.87 ± 1.25, respectively. However, radical scavenging activities were measured as 4.43 ± 0.32, 8.86 ± 0.67, and 14.89 ± 1.16% for ASC and 6.02 ± 0.51, 7.95 ± 0.38, and 10.57 ± 1.23% for BHT, in the same concentrations. To calculate the IC_{50} concentration, the graphic of RSA percentage was drawn and according to the reference graphic, the IC_{50} values of the carotenoid extract of *H. thermotolerans*, ASC, and BHT were calculated as 1.284, 3.617, and 7.912 µg/mL, respectively. There was a statistically significant interaction between the effects of antioxidants and carotenoid extract doses on RSA ($p < 0.05$) (Table 2).

Table 2 Antioxidant efficiency of carotenoid extract *Haloterrigena thermotolerans* K15 compared to commonly use commercial antioxidants

Antioxidants	Dose (µg/mL)	RSA (%)
ASC	0.9	14.89 ± 1.16^{c}
	0.45	8.86 ± 0.67^{de}
	0.09	4.43 ± 0.32^{g}
BHT	0.9	10.57 ± 1.23^{d}
	0.45	7.95 ± 0.38^{ef}
	0.09	6.02 ± 0.51^{fg}
BR	0.9	36.87 ± 1.25^{a}
	0.45	24.27 ± 0.83^{b}
	0.09	8.75 ± 0.70^{de}
Two-way ANOVA *p*-value	Antioxidants	< 0.001
	Dose	< 0.001
	Antioxidants*dose	< 0.001

N = 10, data presented mean ± SD; different lowercase letters indicate interactions between the two factors (antioxidants vs doses)

ASC ascorbic acid; *BHT* butylated hydroxytoluene; *BR* carotenoid extract of *H. thermotolerans* K15

Discussion

Molecules synthesized by halophilic archaea are important for many industrial processes as they adapt to harsh conditions such as extreme salinity and temperature. One of these crucial molecule groups is carotenoid which is preferred because of its antioxidant properties. Hence, carotenoid characterization and determination of the antioxidant activity of the carotenoid extract of *Haloterrigena thermotolerans* K15 isolated from Salt Lake, Turkey, and identified by biochemical and phylogenetic analysis, were performed in the present study. As a result, the selected strain has Gram-negative properties. According to the findings of phylogenetic analysis, the selected strain was similar to *Haloterrigena thermotolerans* PR5 type with a rate of 99.5%. This strain was firstly isolated from the solar salterns in Cabo Rojo, Puerto Rico, in June 1994 (Montalvo-Rodríguez et al. 2000) and from Salt Lake in 2005 by Mutlu (2006). Furthermore, *H. thermotolerans* was isolated also from Seyfe Lake, which is located in the same region of Salt Lake (Ozcan et al. 2007). According to the hypothesis created by Kemp et al. (2018), the microbiota of salt lakes can be transported between sources by the mobility of migratory birds. It is possible to assert that the detection of *H. thermotolerans* in both Turkish lakes could be the result of such carrying activities by animals. *H. thermotolerans* PR5 was reported as a slender rod shape by phase-contrast microscopy at 60 °C incubation temperature and suggested that cell lengths increase due to inhibition of cell division at this temperature (Montalvo-Rodríguez et al. 2000). Therefore, in the present study, cells of *H. thermotolerans* at 40 °C are viewed as coccobacilli by advanced methods of imaging, SEM, and AFM. Antibiotic tests are among the methods that are easy to access and extensively used in the detection of archaea. Albuquerque et al. (2012) reported that halophilic archaea isolates are susceptible to the bacitracin antibiotic; besides, Holmes and Dyall-Smith (1991) reported that the majority of halophilic archaea are susceptible to novobiocin antibiotics as well as bacitracin. In the current study, it was determined that the selected strain was susceptible to novobiocin and bacitracin antibiotics. Montalvo-Rodríguez et al. (2000) reported that *H. thermotolerans* PR5 hydrolyzed Tween 80 and gelatin, but not starch and casein. In addition to this, the same authors reported the reduction of some nitrite from nitrate. In the present study, it was found that *H. thermotolerans* K15 has gelatinase but not amylase activities, just as strain PR5. However, it was able to hydrolyze casein but not Tween 80, and reduction of nitrate to nitrite was not observed, unlike the previously cited study. Although strain PR5 can grow at least 2 mol/L NaCl, K15 required a 1.7 mol/L minimum. Based on this data, it can be thought that strain K15 has a wider tolerance to salt concentration in the growth media.

Carotenoids have an important role in industrial uses such as cosmetics, pharmaceutics, and medical and food processing (Rodrigo-Baños et al. 2015). The most important reason for their intensive use in a different industry is that carotenoids can show high antioxidant, anticancer, and colorant effects, and they provide these effects at the same time (Torregrosa-Crespo et al. 2018; Giani and Martínez-Espinosa 2020). More than 600 carotenoids are known to occur in nature. The carotenoid market is divided into different segments and has been growing rapidly in recent years all over the world. Especially anti-aging products and cosmetic products make up a large part of this market (Galasso et al. 2017). Although chemical synthesis is the most widely used method for carotenoid production, this method has some advantages and disadvantages. Chemical synthesis procedures of carotenoids have been determined primarily for high commercial potential carotenoids such as xanthophylls, astaxanthin, canthaxanthin, and lutein. Despite the observation that carotenoids produced by chemical synthesis ensure high purity product acquisition, the chemical synthesis of some carotenoids is very complex and, as a result, slow and expensive. Thus, the biosynthesis of these carotenoids can be more usable (Naziri et al. 2014). Recent researches have reported that halophilic microorganisms, especially halophilic archaea, are important sources for carotenoid production (Oren 2010). Halophilic archaea are an extremely important source, especially for the production of not chemically synthesized C_{50} carotenoids in particular bacterioruberin and derivatives such as monoanhydrobacterioruberin and bisanhydrobacterioruberin in addition to carotenoids with 40 carbons (Hou and Cui 2018). Membrane stabilization in halophilic archaea is provided by C_{50} carotenoids that have a polar end in their molecules and are suitable for membrane stabilization (Kirti et al. 2014). Bacterioruberin, a C_{50} carotenoid, has four hydroxy groups and functions like a “rivet” in the cell membrane and provides the hardness and mechanical strength of the cell membrane (Lazrak et al. 1988). Also, the effects of bacterioruberin on membrane fluidity ensure the survival of organisms in hypersaline or low-temperature conditions, by acting as a barrier against water and permeability to oxygen and other molecules known (Fong et al. 2001). Besides, bacterioruberin has 13 double-conjugated bonds and thus functions as a hydroxyl free radical scavenger, protecting haloarchaea against oxygen damage, high light intensity, UV rays, and exposure to H_2O_2 (Shahmohammadi et al. 1998; Giani and Martínez-Espinosa 2020).

Squillaci et al. (2017) used four different solvents, namely, methanol, acetone, *n*-hexane, and ethyl acetate for carotenoid extraction of *Haloterrigena turkmenica* and found that methanol was the most effective solvent after two steps of extraction. However, in this study, before the extraction process, the cell pellet was washed with PBS, and after the first extraction, the extract was obtained as dark pink, while the complete color removal of the cell pellet and the characteristic bacterioruberin spectrum were obtained. It is thought that the reason for getting efficient results in the first extraction is that contaminant protein residues have been removed as a result of washing with PBS before extraction. In this way, the loss of carotenoids emerging as a result of the first extraction was prevented.

Both the spots obtained in TLC and the total extract were analyzed, but the peaks obtained from the total extract were examined to prevent the loss that may occur during spot recovery. In addition, when TLC spots are examined, it can be said that there is more than one component in each spot; in other words, several chemically similar components migrate together in the thin layer.

UV spectrum of carotenoids, spectral fine structure, *cis* peak intensity, and previously reported data were evaluated together in the determination of carotenoid contents of the *H. thermotolerans* K15 due to the absence of reference standard materials in which the obtained carotenoid types can be compared with chromatographic methods and the mass spectrometric analysis is insufficient to reveal the differences between geometric isomers.

According to the results, all-trans bacterioruberin (All E BR) was identified as a major carotenoid for *H. thermotolerans* K15, monoanhydrobacterioruberin (MABR), and bisanhydrobacterioruberin (BABR), and their isomers were also detected within the limits of the methods used. Among the fourteen peaks obtained on the chromatogram of the total extract, it is clear that the first peak, All E BR, has maximum intensity. Naziri et al. (2014) analyzed the carotenoid production of *Halorubrum* sp. TBZ126 and detected bacterioruberin, lycopene, and β-carotene by chromatographic and mass spectrometric analysis, but there was no detailed data on the BR isomers. The carotenoid characterizations of *Haloterrigena turkmenica* and *Haloterrigena* sp. strain SGH1 have been reported and found that all-trans bacterioruberin is the major carotenoid, and many geometric isomers of the bacterioruberin were also detected (Squillaci et al. 2017; Flores et al. 2020).

The conjugated double-bond system gives the carotenoid molecules a robust and linear skeletal structure while providing a high reduction potential that makes these molecules potential antioxidants. The effect of carotenoids as antioxidants is evaluated by reacting with oxidizing agents and peroxy radicals. These properties play an important role in the stabilization of carotenoids (Gruszecki and Strzałka 2005). The antioxidant properties of carotenoids are directly related to their molecular structure. The scavenging capabilities of carotenoids are influenced by two basic structural features, namely, the length of conjugated double-bond numbers and whether a chemical group is attached to its structure. Therefore, bacterioruberin with 13 conjugated double bonds shows higher antioxidant activity than astaxanthin with 11 conjugated double bonds and lutein with ten conjugated double bonds. At the same time, while there is a hydroxyl group in both terminal rings in the structure of astaxanthin and lutein, the presence of four hydroxyl groups in the bacterioruberin is considered another reason for its high antioxidant activity (Yachai 2009). DPPH assay of the present study clearly showed that the antioxidant ability of extracted carotenoid from *H. thermotolerans* (BR) was significantly higher ($p < 0.05$) than other extensively used commercial antioxidants such as ascorbic acid (ASC) and butylated hydroxytoluene (BHT). Yachai (2009) reported that DPPH radical scavenging activity increased with the increasing concentration of carotenoid isolated from *Halobacterium salinarum*, an extremely halophilic archaeon. The antioxidant activity of bacterioruberin also compared the antioxidant activities of lutein, astaxanthin, β-carotene, and lycopene. According to the results of the study, the bacterioruberin had the lowest IC_{50} value, in other words, the highest antioxidant activity, and this was related to the presence in bacterioruberin of 13 double-conjugated double bonds. In this context, the fact that the major carotenoid extracted from the isolated species in our study was bacterioruberin explains the high antioxidant activity obtained. In addition, the increase in antioxidant activity with increasing carotenoid concentration supports the data of the study.

In conclusion, an extremely halophilic and thermophilic archaeon, *Haloterrigena thermotolerans* K15 isolated from Salt Lake-Turkey, has a great potential for bacterioruberin-rich carotenoid content, and the extracted carotenoid has more antioxidant effect than commonly used chemical antioxidants like ascorbic acid and butylated hydroxytoluene. The results suggest the potential use of *H. thermotolerans* K15 products in different sectors, as a substitute for synthesized chemical carotenoids and antioxidants.

Author contributions FIK: Conceived and designed research, conducted experiments, analyzed data, and wrote the manuscript. NG: Supervision, reviewing, and editing. All authors read and approved the manuscript.

Data availability All data are available upon reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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